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13. ABSTRACT ***(Maximum 200 Words)***

We hypothesize that chromosomal loss of the Y chromosome in prostate cancer is a specific tumorigenic event. Transfer of the Y chromosome by microcell mediated chromosome transfer into PC-3 cells resulted in suppression of tumor growth. However, these PC-3 hybrids containing the Y chromosome did grow in soft agar indicating a lack of correlation between *in vitro* and *in vivo* tumor growth. We have isolated clones with fragments of the Y chromosome and found suppression of tumorigenesis correlates with either the short arm of the Y or two small regions on the q arm. The data from the functional assay will be correlated with data from deletions in patient material. We have isolated a complete genomic microarray specific for the Y chromosome. These BAC (bacterial artificial chromosomes) will be used to perform array CGH (Comparative genomic hybridization) of prostate tumor samples. Successful identification of a gene on the Y chromosome will provide a marker that may aid in the diagnosis and prediction of prognosis of prostate cancer.

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INTRODUCTION:

Loss of the Y chromosome has been noted in prostate tissue by other investigators [Hum. Pathol. 27:720(1996); Cancer Genet. Cytogent. 66:93(1993); and Cancer Res. 54:4472(1994)]. These experiments have not been able to resolve whether a specific region of the Y chromosome is deleted in prostate cancer. Chromosomal loss is a hallmark of a tumor suppressor gene. However, functional proof for a tumor suppressor gene comes from experiments using microcell transfer of a human Y chromosome with a selectable marker. Our data to date have demonstrated that insertion of the Y chromosome into the prostate cancer line PC-3 results in the suppression of tumor growth in nude mice. Our studies are designed to locate the region of the gene causing tumor suppression by the Y chromosome. We will correlate these data with data indicating loss of the Y chromosome in patient samples. The combination of the laboratory functional analysis with the analysis of the patient samples should indicate the location of a tumor suppressor gene on the Y chromosome and facilitate the cloning of this gene.

BODY:

During the past year we have concentrated on the characterization of the hybrids formed from the introduction of the Y chromosome into PC-3 cells. In addition we have isolated all the reagents needed to make a complete tiling path of the BAC (bacterial artificial chromosome) clones for the Y chromosome. We have enlisted the company Spectral Genomics to print our slides. Our third major area of work has been to make a subtractive library to identify candidate genes on the Y chromosome.

Task 1. PCR analysis of tumor samples, Months 1-15

Patient samples

Over 300 patient samples have been collected an entered into the repository. We have isolated DNA from frozen tissue.

Y chromosome specific markers (PCR (polymerase chain reaction) assays)

We have continued to perfect a reliable panel of Y chromosome-specific markers. Currently we are using a panel of 34 markers that are assayed by PCR.

Analyze tumors with Y specific markers

After considering the problem of normal cell contamination in prostate tumors, it has become clear that PCR of samples may be difficult to interpret. Quantitative assays such as real time PCR would be an option if they were not so expensive. Consequently, we have decided to use the genomic array analysis of tissue until we can establish the critical region.

Task 2. In situ hybridization of interphase tumor cells, Months 3-15

Develop Y chromosome- specific probes for in situ

In isolating BAC clones for genomic arrays, we have identified clones that are firmly placed on the Y chromosome. We have also examined each clone for repetitive sequences as well as sequences on other chromosome. These clones are currently being evaluated as in situ hybridization probes

Task 3. Genomic DNA array, Months 1-15

Develop Y chromosome specific clones for DNA arrays

As the genome project has developed so has the availability of clones for specific chromosomes. Although we have used the clones we isolated previously, we were able to take advantage of the fact that a minimum tiling path for the Y chromosome is available. By choosing the clones that are included in this path we will have coverage of the 22.8 Mb of euchromatic DNA on the Y chromosome. Although the Y is considered to be 58.4 Mb, the rest of the sequence is repetitive DNA. Each clone from the path was subcloned. Each clone was tested with a PCR primer specific for the BAC clone. Some of the primers were for markers that had previously been mapped to the clone while others were newly derived from the sequence. Previously we had identified clones for 71 markers. Our coverage now is much better - at approximately three times the density as before. Several clones were eliminated because they contained too many repetitive sequences. Other clones were not positive for markers by PCR. A summary of all the data we have generated is in Table 1.

To produce BAC arrays, a large amount of DNA must be isolated for each clone. This process is quite laborious and has taken a great deal of time to isolate sufficient quantities of DNA to do all of our experiments.

Establish conditions for stamping arrays and hybridization

We spent quite a bit of time trying to optimize the conditions for producing arrays. The quality of our "homemade" arrays was at best spotty. Consequently, we have enlisted the help of Spectral Genomics in Houston to spot slides for us and to share their protocol for hybridization. As we have worked with them on other projects, we are certain that this approach will be more successful and faster than for us to make our own slides. Array CGH (comparative genomic hybridization) will allow us to quantitate Y chromosome sequences as well as precisely locate them on the chromosome.

Task 4. Microcell transfer of Y chromosome fragments, Months 4-18

In our original experiments we transferred three independently marked Y chromosomes from the rodent background into PC-3 cells. Hybrids containing the Y chromosome were no longer capable of forming tumors in nude mice. During the course of this year we discovered that the Y chromosome was transferred from the Chinese hamster – Y chromosome hybrid, not the mouse – Y chromosome hybrid. The person who made the hybrids had left the laboratory and the assumption that he had used the mouse hybrids was made. After checking back in his records and checking the DNA, we concluded that the marked Y chromosome had been transferred from the Chinese hamster background. We tested the Chinese hamster parental line and found that it did not make tumors in nude mice. Consequently our plans to bypass PC-3 hybrids by using the rodent hybrids in searching for the smallest region were abandoned.

The second finding that changed our approach was the data on soft agar growth. Although the PC-3 hybrids did not make tumors in nude mice, they did grow well in soft agar (Figure). To further test the cells, we isolated clones of PC-3 hybrids that had grown in soft agar. Three of the original PC-3 hybrids (2-2C1, 2-2C12, and 1-5BE) were grown in soft agar and then individual clones isolated. Of the 28 clones that were isolated, we injected 9 into nude mice. These clones still did not form tumors in nude mice with the exception of one clone. DNA marker analysis was performed to determine if the Y chromosome were still intact. Several of the clones had lost part of the Y chromosome and yet were suppressed for tumorigenesis.

Transfer the Y chromosome fragments into PC-3 cells

Two strategies are being taken to isolate fragments of the Y chromosome. One arose from our studies with soft agar clones. Several of the clones had lost pieces of the Y chromosome and were studied as described above. Other isolates are also available for study. They will be assayed for markers and if portions of the Y are lost, they will be injected into nude mice.

The second strategy we are taking is more directed. Since our current data point to the short arm of the Y chromosome as the most likely location of this gene, we are making directed deletions of the chromosome. To do this we are using a Cre/lox system. The details of this experiment are shown in Figure. Basically, two plasmids are inserted at separate sites on the short arm of the Y chromosome. These plasmids each contain half of the HPRT (hypoxanthine phosphoribosyl transferase) gene. By using a transient transfection of bacterial Cre recombinase, the DNA will recombine to exclude the Y chromosome sequences in between the two plasmids and to make an intact HPRT gene. The correct recombinants containing deletions of the Y chromosome will be selected on HAT medium. These directed constructs will then be transferred into PC-3 cells and tested in functional assays.

Isolate independent clones and assay for Y specific markers

We have accomplished this goal on our soft agar subclones. We will characterize our directed deletions in a like manner.

Perform in vitro assays for tumor growth

We have tested the clones in soft agar as explained above. We will characterize our directed deletions in a like manner.

Inject tumors into nude mice and quantitate tumor growth

Subclones that were obtained from soft agar have been injected into nude mice. We will characterize our directed deletions in a like manner.

Task 5. Candidate gene identification, Months 18-24

Isolate candidate cDNAs

We used two strategies to isolate candidates. First, we took advantage of the fact that a sincle tumor grew out of the line 2-6 E2. We cultured the tumor cells for further use. Another line 2-6A3 never formed tumors in nude mice. Since they originated from the same marked Y chromosome, we decided these clones would be candidates for subtractive hybridization. We looked for clones that were expressed in 2-6A3, but not 2-6E2 and vice versa. The libraries obtained were assessed for inserts and plated into the 96 well format. The clones were hybridized with RNA from 2-6A3 and 2-6E2 to identify sequences that were differentially expressed.

Three dozen of the most promising clones were sequenced. Most of the clones corresponded to genes that were identified in GenBank, but a few were unique sequences. Many of the clones were Chinese hamster indicating that a small amount of Chinese hamster DNA had been transferred with the Y chromosome and that this DNA was different in each hybrid cell. After each clone was identified, the RNA abundance in the 2-6E2 and 2-6A3 hybrids was determined

by either RT-PCR (reverse transcriptase polymerase chain reaction) or by northern blot. The results of this experiment are in Table 3. Although we obtained sequences that were differentially expressed, none of the genes isolated appear to be the gene on the Y chromosome responsible for tumor suppression.

As most of the unique sequences on the Y chromosome have been obtained, we are closely monitoring the genes that are identified in the region of the Y that correlated with tumor suppression. Any likely candidate will be examined further.

Task 6. Prepare manuscripts and final report, Months 20-24
We have prepared the first manuscript reporting the suppression of tumor formation of PC-3 by the Y chromosome (see appendix).

KEY RESEARCH ACCOMPLISHMENTS:

- In situ hybridization of the HisD gene confirmed the location of the selectable marker at the end of the p arm of the Y chromosome
- Introduction of the Y chromosome into PC-3 prostate cancer cells results in the suppression of tumor growth in nude mice
- The pTKm3 hybrids were confirmed as being Chinese hamster and 15 somatic cell hybrids containing fragments of the human Y chromosome were characterized for additional markers
- 38 microcell PC-3 hybrids identified with fragments of the Y chromosome were characterized with additional markers bringing the total to 34 markers
- Introduction of the Y chromosome into PC-3 prostate cancer cells does not change the growth of these cells in soft agar
- Subclones (28) that grow on soft agar were isolated from three of the PC-3 hybrid clones
- Nine soft agar-derived clones were injected into nude mice and only 1 of the subclones grew tumors in nude mice
- Soft agar subclones were characterized for 34 markers on the Y chromosome
- The region of the Y chromosome associated with the suppression of tumor growth has been limited to the short arm and two small regions of the long arm of the Y chromosome
- Plasmid constructs have been made for directed deletions on the p arm of the Y chromosome
- The genomic array of BAC clones for the Y chromosome has been confirmed by PCR for Y specific markers
- DNA has been isolated from 161 BAC clones in preparation for spotting onto glass slides by Spectral Genomics. Twenty one clones remain for the isolation of DNA.
- DNA has been isolated from frozen tumor samples
- Prostate tumor samples have been collected and patient data maintained in a database for >300 patients A subtractive library was constructed to identify possible candidates

REPORTABLE OUTCOMES:

- Abstract presented at the Cold Spring Harbor Meeting on Cancer Genetics and Tumor Suppressor Genes in August, 2002

- Graduate student presentation of the data at the Texas Genetics Society Meeting, April, 2002
- Manuscript on the introduction of the Y chromosome to PC-3 (see appendix)
- 182 BAC clones for the length of the Y chromosome euchromatic region have been isolated and verified. These verified clones are available to the community.

CONCLUSIONS:

We have shown that the introduction of the Y chromosome into PC-3 results in the suppression of tumor growth in nude mice. Surprisingly, the insertion of the Y chromosome does not inhibit growth in soft agar. By studying clones with fragments of the Y the gene responsible for suppression appears to be either on the short arm or on two small regions of the long arm. We have nearly in place all the genomic clones to make an array that will be used in aCGH (array comparative genomic hybridization) to detect deletions in prostate tumor samples. Since we have developed a complete tiling path for the Y chromosome, we expect to be able to precisely locate deletions. With BACs the resolution will be within 50 Kb. Consequently, we will be able to correlate our laboratory functional data with patient material. Our ultimate goal will be to clone the gene that codes for the tumor suppression activity.

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Baretton GB, Valina C, Vogt T, Schneiderbanger K, Diebold J, Lohrs U. (1994) Interphase cytogenetic analysis of prostatic carcinomas by use of nonisotopic in situ hybridization. Cancer Res. 54:4472-80.

APPENDICES

Table 1. Characterization of BAC clones for a human genomic array of the Y chromosome
Table 2. Marker content of soft agar clones
Table 3. Summary of clones from subtractive libraries
Figure 1. Soft agar growth of PC-3 hybrids
Figure 2. Tumor growth in nude mice of soft agar-selected clones
Figure 3. Strategy for producing directed deletions of the Y chromosome
Figure 4. Summary of the smallest region for the location of the tumor suppressor gene
Manuscript "Y chromosome suppresses the tumorigenicity of PC-3, a human prostate cancer cell line, in athymic nude mice"

Table 1
PCR Verification for Y Chromosome BAC Clones

Path	Accession No.	BAC Clone	Marker Tested	Result	DNA
1	AC006040.3	RP11-400O10	SRY	POSITIVE	YES
2	AC074181.1	RP11-515L2	515L2*	NEGATIVE	NO
3	AC006157.2	RP11-414C23	ZFY	POSITIVE	YES
4	AC006032.2	RP11-115E20	DXYS106	POSITIVE	YES
5	AC006152.3	RP11-4N7	DYS 395	POSITIVE	YES
6	AC011305.2	RP11-390E9	sY 721	POSITIVE	YES
7	AC009479.4	RP11-278L6	sY 870	POSITIVE	YES
8	AC019058.4	RP11-125B15	sY 872	POSITIVE	YES
9	AC024038.6	RP11-349O6	AF20109	POSITIVE	YES
10	AC012078.3	RP11-539O22	539O22*	POSITIVE	YES
11	AC010094.5	RP11-336O5	sY 703	POSITIVE	YES
12	AC010737.4	RP11-439L24	DXYS112	POSITIVE	YES
13	AC010084.3	RP11-145J12	DYS 253	POSITIVE	YES
14	AC010905.3	RP11-560B8	560B8*	POSITIVE	NO
15	AC010106.2	RP11-575J5	To streak		
16	AC024703.5	RP11-51N20	51N20*	POSITIVE	YES
17	AC012077.4	RP11-524G14	sY 876	POSITIVE	YES
18	AC010142.4	RP11-240N18	sY 875	POSITIVE	YES
19	AC019060.5	RP11-125K5	sY 1008	POSITIVE	YES
20	AC023423.5	RP11-430C23	sY 936	POSITIVE	YES
21	AC010722.2	RP11-122L9	sY 2138	POSITIVE	YES
22	AC010685.3	RP11-465A8	DYS 255	POSITIVE	YES
23	AC010129.3	RP11-59N9	sY 2141	POSITIVE	YES
24	AC012067.2	RP11-192N14	sY 2146	POSITIVE	YES
25	AC012667.2	RP11-357C22	sY 716	POSITIVE	YES
26	AC010081.4	RP11-65E7	DYS 256	POSITIVE	YES
27	AC010874.3	RP11-118K2	118K2*	POSITIVE	YES
28	AC010977.4	RP11-362J16	sY 2171	POSITIVE	YES
29	AC016681.2	RP11-62H15	sY 866	POSITIVE	YES
30	AC010140.3	RP11-218E11	sY 1011	POSITIVE	YES
31	AC006335.2	RP11-492C2	DYS 379	POSITIVE	YES
32	AC010154.3	RP11-573O23	DYS 257	POSITIVE	YES
33	AC010144.4	RP11-309M4	sY 1091	POSITIVE	YES
34	AC010728.4	RP11-258E22	258E22*	POSITIVE	YES
35	AC013412.3	RP11-507A3	507A3*	POSITIVE	N0
36	AC011297.3	RP11-115H13	DYS 266	POSITIVE	YES
37	AC012068.5	RP11-196J6	sY 2234	POSITIVE	YES
38	AC010104.3	RP11-540C18	DXS7855	POSITIVE	YES
39	AC010143.3	RP11-301O17	sY 887	POSITIVE	YES
40	AC007284.4	RP11-558K21	To streak		
41	AC007247.5	RP11-305H21	DYS 261	POSITIVE	YES
42	AC007274.3	RP11-105L10	DYS 260	POSITIVE	YES
43	AC007275.4	RP11-109F19	DYS 288	POSITIVE	YES
44	AC010678.4	RP11-108F14	DYS 54	POSITIVE	YES
45	AC010902.4	RP11-549J7	549J7*	NEGATIVE	
46	AC016749.4	RP11-504E20	SHGC-107423	POSITIVE	YES
47	AC051663.9	RP11-475P15	sY 1103	POSITIVE	YES
48	AC025731.12	RP11-48H21	48H21*	POSITIVE	YES
49	AC016991.5	RP11-17E15	17E15*	POSITIVE	YES
50	AC064829.6	RP11-375P13	sY 953	POSITIVE	YES
51	AC009491.3	RP11-418M8	DYS 231	POSITIVE	YES
52	AC007967.3	RP11-373F14	SHGC-80640	POSITIVE	YES
53	AC068719.3	RP11-403P11	sY 894	POSITIVE	YES
54	AC079126.3	CTB-45E23	REPEATS		

112	AC007043.3	RP11-507E21	sY 2545	POSITIVE	YES
113	AC006999.2	RP11-462A19	DYS 201	POSITIVE	YES
114	AC007042.3	RP11-399H17	sY 2568	POSITIVE	YES
115	AC091329.3	RP11-568H21	To streak		
116	AC007972.4	RP11-537C24	DYS 202	POSITIVE	YES
117	AC015978.4	RP11-529I21	DYS 241	POSITIVE	YES
118	AC068704.4	RP11-434F12	DYS 203	POSITIVE	YES
119	AC007742.4	RP11-357E16	DYS 211	POSITIVE	YES
120	AC095381.1	GAP1623	Not BAC clone		
121	AC009976.4	RP11-509B6	DYS 241	POSITIVE	YES
122	AC095380.1	GAP1622	Not BAC clone		
123	AC024183.4	RP11-268K13	To streak		
124	AC007241.3	RP11-157F24	DYS 203	POSITIVE	NO
125	AC069130.6	RP11-468D10	DYS 241	POSITIVE	YES
126	AC073962.5	RP11-945E12	945E12*	POSITIVE	YES
127	AC068541.7	RP11-243P9	DYS 211	POSITIVE	YES
128	AC022486.4	RP11-569J3	DYS 208	POSITIVE	YES
129	AC007379.2	RP11-143C1	DYS 208	POSITIVE	YES
130	AC009235.4	RP11-392F24	DYS 212	POSITIVE	YES
131	AC007244.2	RP11-207L19	DYS 213	POSITIVE	YES
132	AC021210.4	RP11-389F23	sY 919	POSITIVE	YES
133	AC010133.4	RP11-118E9	sY 916	POSITIVE	YES
134	AC012062.4	RP11-80E19	sY 2608	POSITIVE	YES
135	AC010137.3	RP11-169D1	DYS 214	POSITIVE	YES
136	AC009977.4	RP11-576C2	sY 2615	POSITIVE	YES
137	AC010889.3	RP11-424G14	sY 971	POSITIVE	YES
138	AC010151.3	RP11-508P10	sY 969	POSITIVE	YES
139	AC009233.3	RP11-356K22	DYS 217	POSITIVE	YES
140	AC079157.3	RP11-1285C3	1285C3*	POSITIVE	YES
141	AC079261.2	RP11-1325K3	REPEATS		
142	AC079156.3	RP11-943F15	sY 1155	POSITIVE	YES
143	AC024250.6	RP11-684N2	sY 1155	POSITIVE	YES
144	AC009240.6	RP11-489O13	489O13*	POSITIVE	NO
145	AC011745.4	RP11-329C15	DYS 392	POSITIVE	YES
146	AC007678.3	RP11-256K9	DYS 219	POSITIVE	YES
147	AC009494.2	RP11-450B24	450B24*	POSITIVE	NO
148	AC026061.8	RP11-223K9	223K9*	NEGATIVE	
149	AC009489.3	RP11-339J4	sY 1013	POSITIVE	YES
150	AC007876.2	RP11-65G9	DYS 221	POSITIVE	YES
151	AC009239.3	RP11-470K20	470K20*	NEGATIVE	
152	AC010086.4	RP11-209I11	New primers		
153	AC010141.2	RP11-220O2	DYS 225	POSITIVE	YES
154	AC021107.3	RP11-178M5	DYS 258	POSITIVE	YES
155	AC078938.3	CTC-480L15	To streak		
156	AC024236.5	RP11-400I17	DYS 230	POSITIVE	YES
157	AC007322.4	RP11-553C13	DYS 400	POSITIVE	NO
158	AC007359.3	RP11-66M18	DYS 379	POSITIVE	YES
159	AC023342.3	RP11-95B23	DYS 77	POSITIVE	YES
160	AC025227.6	RP11-109G18	DYS 227	POSITIVE	YES
161	AC007320.3	RP11-477B5	DYS 77	POSITIVE	YES
162	AC008175.2	RP11-427G18	SHGC-7605	POSITIVE	YES
163	AC016694.2	RP11-123G1	123G1*	POSITIVE	YES
164	AC010080.2	RP11-5C5	sY 990	POSITIVE	YES
165	AC016911.6	RP11-473E1	473E1*	POSITIVE	YES
166	AC006366.3	RP11-86G22	DYS 235	POSITIVE	YES
167	AC010088.3	RP11-289L7	sY 2716	POSITIVE	YES
168	AC053490.2	RP11-140H23	DYS 236	POSITIVE	YES

55	AC079125.4	RP11-1188O8	DYS 392	POSITIVE	YES
56	AC009952.4	RP11-175I4	DYS 258	POSITIVE	YES
57	AC025732.9	RP11-116J19	116J19*	POSITIVE	YES
58	AC006158.6	RP11-441G8	sY 1079	POSITIVE	YES
59	AC006156.5	RP11-344D2	DYS 398	POSITIVE	YES
60	AC025819.7	RP11-370N2	370N2*	POSITIVE	YES
61	AC017019.3	RP11-182H20	DYS 379	POSITIVE	YES
62	AC010891.2	RP11-453C1	453C1*	POSITIVE	YES
63	AC006986.3	RP11-155J5	DYS 268	POSITIVE	YES
64	AC006987.2	RP11-160K17	DYS 269	POSITIVE	YES
65	AC010970.3	RP11-108I14	sY 2267	POSITIVE	YES
66	AC069323.5	RP11-1126J10	1126J10*	NOT WORKING	
67	AC011293.5	RP11-75F5	DYS 270	POSITIVE	YES
68	AC012502.3	RP11-461H6	461H6*	POSITIVE	YES
69	AC011302.3	RP11-333E9	DYS 271	POSITIVE	YES
70	AC013735.5	RP11-558M10	558M10*	POSITIVE	NO
71	AC004772.2	CTB-144J1	To streak		
72	AC005942.2	CTC-298B15	To streak		
73	AC002992.1	203M13	RP11 LIBRARY- NEGATIVE		
74	AC004617.2	264M20	RP11 LIBRARY- NEGATIVE		
75	AC004810.1	CTB-69H8	To streak		
76	AC002531.1	486O2	RP11 LIBRARY- NEGATIVE		
77	AC004474.1	475I1	RP11 LIBRARY- NEGATIVE		
78	AC006565.4	CTC-484O7	To streak		
79	AC005820.1	CTC-494G17	To streak		
80	AC010877.3	RP11-218F6	sY 2366	POSITIVE	YES
81	AC006376.2	RP11-386L3	DYS 276	POSITIVE	YES
82	AC007004.3	RP11-521D3	521D3*	NEGATIVE	
83	AC006383.2	RP11-498H20	sY 2375	POSITIVE	YES
84	AC006371.2	RP11-304C24	DYS 277	POSITIVE	YES
85	AC006370.2	RP11-292P9	DYS 246	POSITIVE	YES
86	AC018677.3	RP11-264A13	sY 2395	POSITIVE	YES
87	AC010720.4	RP11-53K10	sY 2384	POSITIVE	YES
88	AC010723.3	RP11-139C10	DYS 227	POSITIVE	YES
89	AC019191.4	RP11-312H22	312H22*	POSITIVE	YES
90	AC010726.4	RP11-224C16	DYS 280	POSITIVE	YES
91	AC010979.3	RP11-384N21	sY 882	POSITIVE	YES
92	AC010879.2	RP11-235I1	sY 2386	POSITIVE	YES
93	AC017032.3	RP11-292E8	sY 910	POSITIVE	YES
94	AC006989.3	RP11-225B4	SHGC-83159	POSITIVE	YES
95	AC011289.4	RP11-59K8	DYS 390	POSITIVE	YES
96	AC010972.3	RP11-133D3	sY 863	POSITIVE	YES
97	AC007007.3	RP11-551F5	sY 2478	POSITIVE	YES
98	AC006998.3	RP11-458M9	DYS 282	POSITIVE	YES
99	AC006382.3	RP11-494J4	DYS 281	POSITIVE	YES
100	AC006462.3	RP11-389B19	sY 2458	POSITIVE	YES
101	AC006336.4	RP11-508K5	sY 770	POSITIVE	YES
102	AC016671.3	RP11-12J24	REPEATS		
103	AC017020.4	RP11-185K15	SHGC-60455	POSITIVE	YES
104	AC011749.2	RP11-455E3	SHGC-78944	POSITIVE	YES
105	AC053516.10	RP11-442J5	sY 2544	POSITIVE	YES
106	AC010135.3	RP11-128D13	DYS 200	POSITIVE	YES
107	AC010128.3	RP11-15H4	New primers		
108	AC011751.2	RP11-478I15	DYS 289	POSITIVE	YES
109	AC016678.4	RP11-55O11	DYS 243	POSITIVE	YES
110	AC015979.4	RP11-538M13	DYS 200	POSITIVE	YES
111	AC007034.4	RP11-99M1	SHGC-5485	POSITIVE	YES

169	AC007039.6	RP11-263A15	263A15*	POSITIVE	YES
170	AC006983.4	RP11-70G12	SHGC-1348	POSITIVE	YES
171	AC009947.2	RP11-39P20	DYS 12	POSITIVE	YES
172	AC016707.2	RP11-221K4	221K4*	POSITIVE	YES
173	AC016752.2	RP11-506M9	SHGC-9458	POSITIVE	YES
174	AC025246.6	RP11-589P14	To streak		
175	AC073649.3	RP11-823D8	New primers		
176	AC073893.4	RP11-978G18	sY 707	POSITIVE	YES
177	AC068601.8	RP11-1067I16	sY 710	POSITIVE	YES
178	AC023274.2	RP11-307L15	307L15*	POSITIVE	NO
179	AC012005.4	RP11-533E23	SHGC-104362	POSITIVE	YES
180	AC013465.4	RP11-424J12	424J12*	POSITIVE	YES
181	AC016698.3	RP11-160O2	DYS 235	POSITIVE	YES
182	AC010153.3	RP11-535I13	REPEATS		
183	AC025735.4	RP11-214M24	sY 2716	POSITIVE	YES
184	AC010089.4	RP11-290O3	sY 579	POSITIVE	YES
185	AC006982.3	RP11-26D12	SHGC-35663	POSITIVE	YES
186	AC006338.5	RP11-539D10	DYS 236	POSITIVE	YES
187	AC016728.4	RP11-363G6	DYS 235	POSITIVE	YES
188	AC006386.4	RP11-566H16	DYS 237	POSITIVE	YES
189	AC006328.5	RP11-102O5	sY 2729	POSITIVE	YES
190	AC007562.4	RP11-497C14	sY 710	POSITIVE	YES
191	AC010682.2	RP11-251M8	sY 707	POSITIVE	YES
192	AC017005.6	RP11-100J21	DYS 241	POSITIVE	YES
193	AC007965.3	RP11-245K4	SHGC-9458	POSITIVE	YES
194	AC006991.3	RP11-270H4	SHGC-1348	POSITIVE	YES
195	AC024067.4	RP11-487K20	DYS 247	POSITIVE	YES
196	AC013734.4	RP11-557B9	DYS 247	POSITIVE	YES
197	AC019099.6	RP11-428D10	sY 1072	POSITIVE	YES
198	AC073880.5	RP11-1136L22	REPEATS		
199	AC068123.5	RP11-242E13	REPEATS		
200	AC025226.4	RP11-57J19	SHGC-7991	POSITIVE	YES

* Custom-made primers

TOTAL **200**
EXCLUDED **18**
FINAL **182**

DNA ISOLATED **161**

To isolate DNA = 7
Clones to streak = 11
New primers required = 3
DNA preps remaining = 21

Table 2. Y Chromosome Markers Tested in the PC-3 Hybrids that Grew in Soft Agar

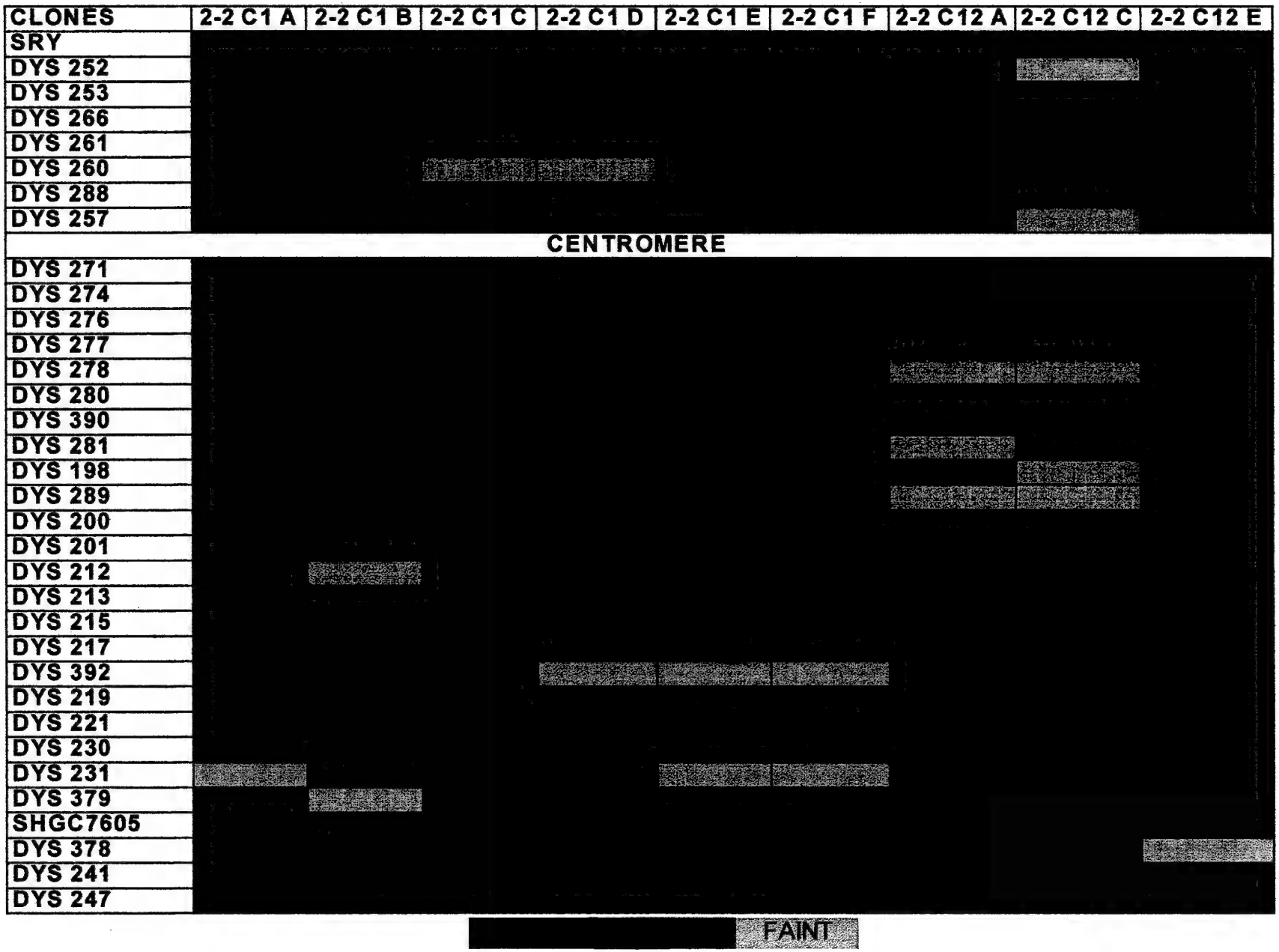


Table 3

DNA sequences identified by subtraction libraries

Genes isolated >one time	human ribosomal protein RPl7A ribosomal protein RPL19 (Chinese hamster) ferritin (Chinese hamster) vimentin (Chinese hamster) calcium binding protein A6 (Chinese hamster)
Human genes found once	NAD dehydrogenase Hs. FLJ2026
Chinese hamster genes found once	laminin receptor poly A binding protein 12S ribosomal CREM transcription factor clone 42 inducible by steroid tubulin saposin heat shock protein HSP70 HMG box protein ribosomal protein S4 stearoyl CoA desaturase
Uncertain species	MEA male specific antigen cathepsin D annexin II
NEW Genes	6

Figure 1A. Growth of PC-3 hybrids on soft agar

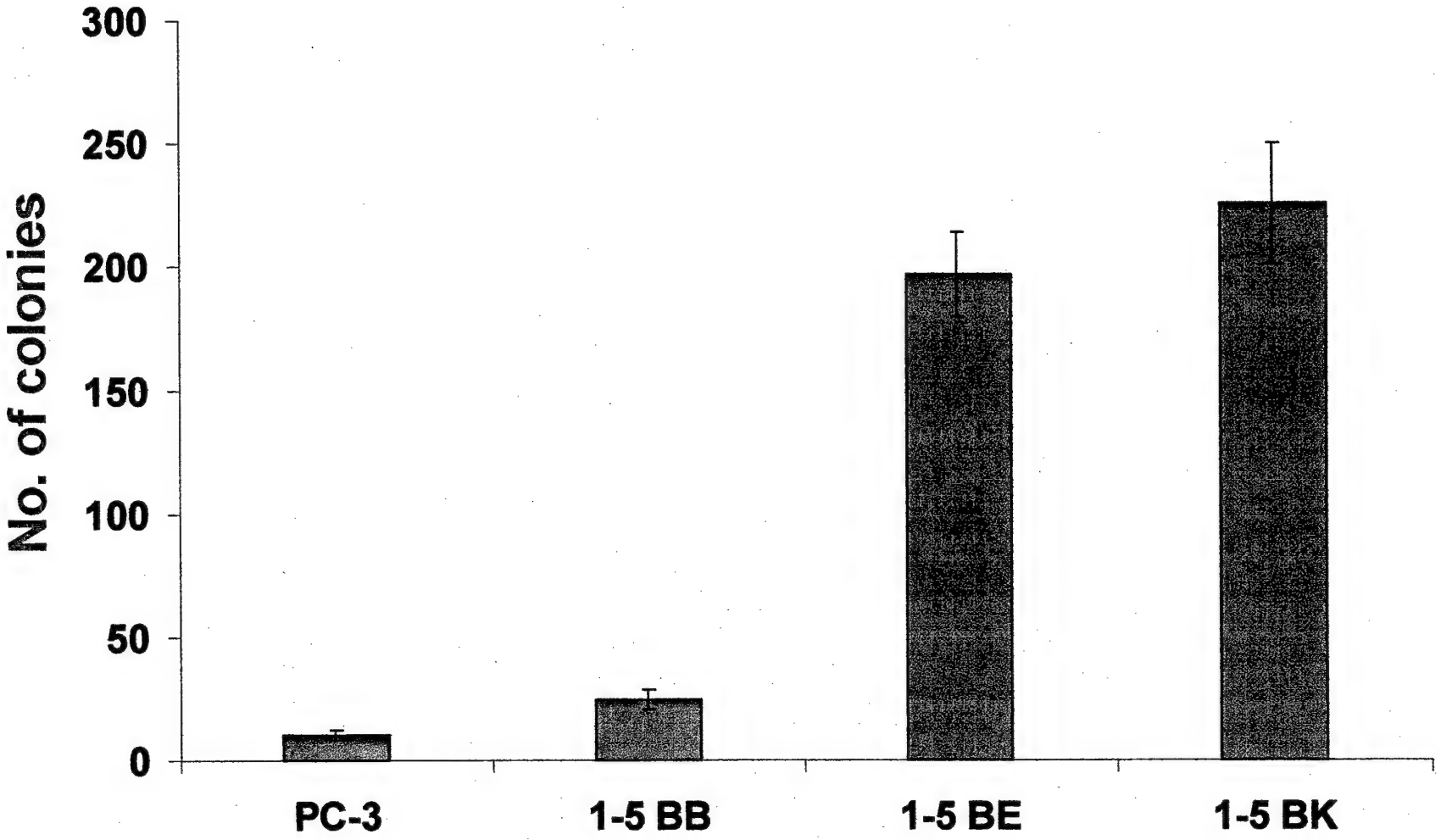


Figure 1B. Growth of PC-3 hybrids on soft agar

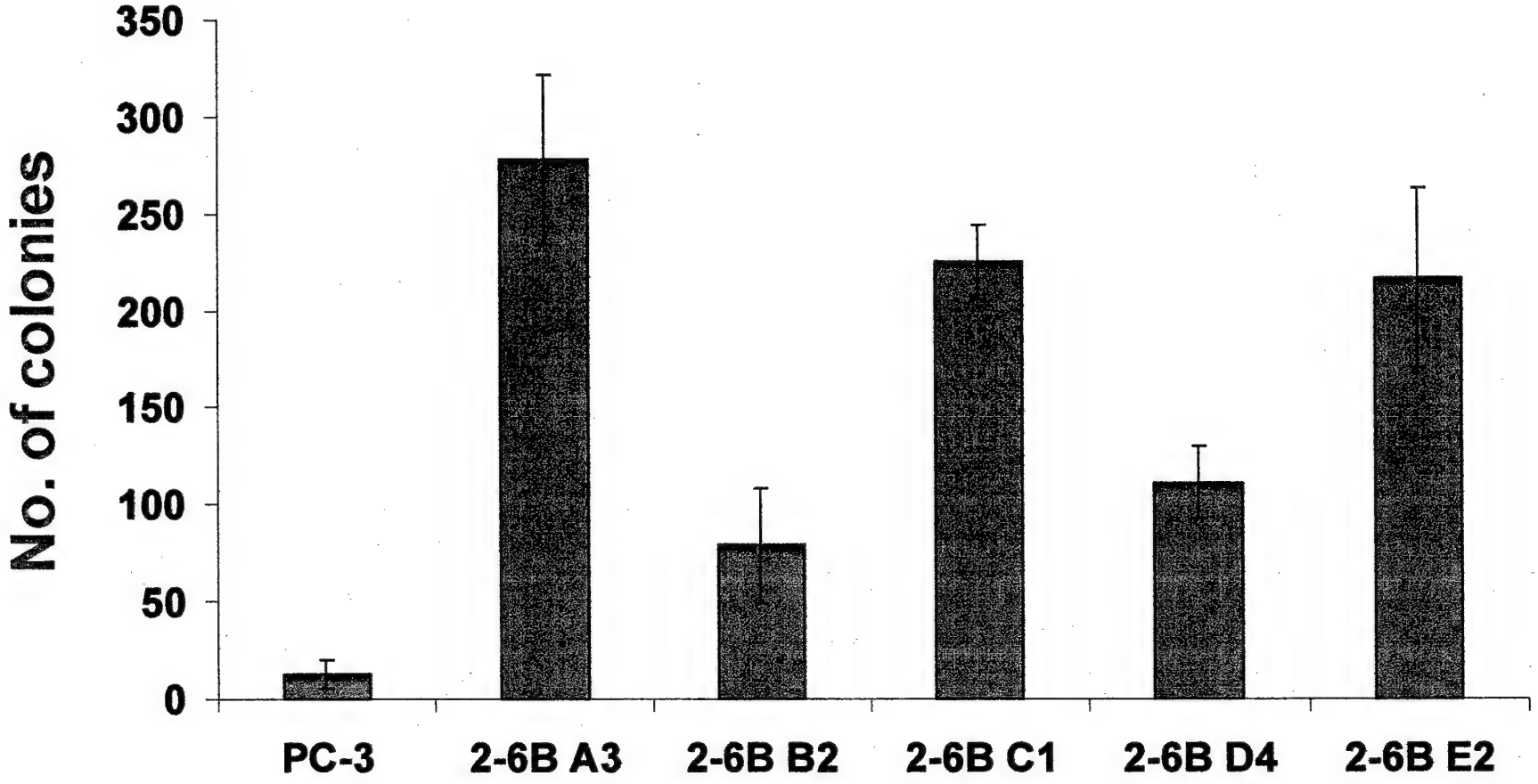
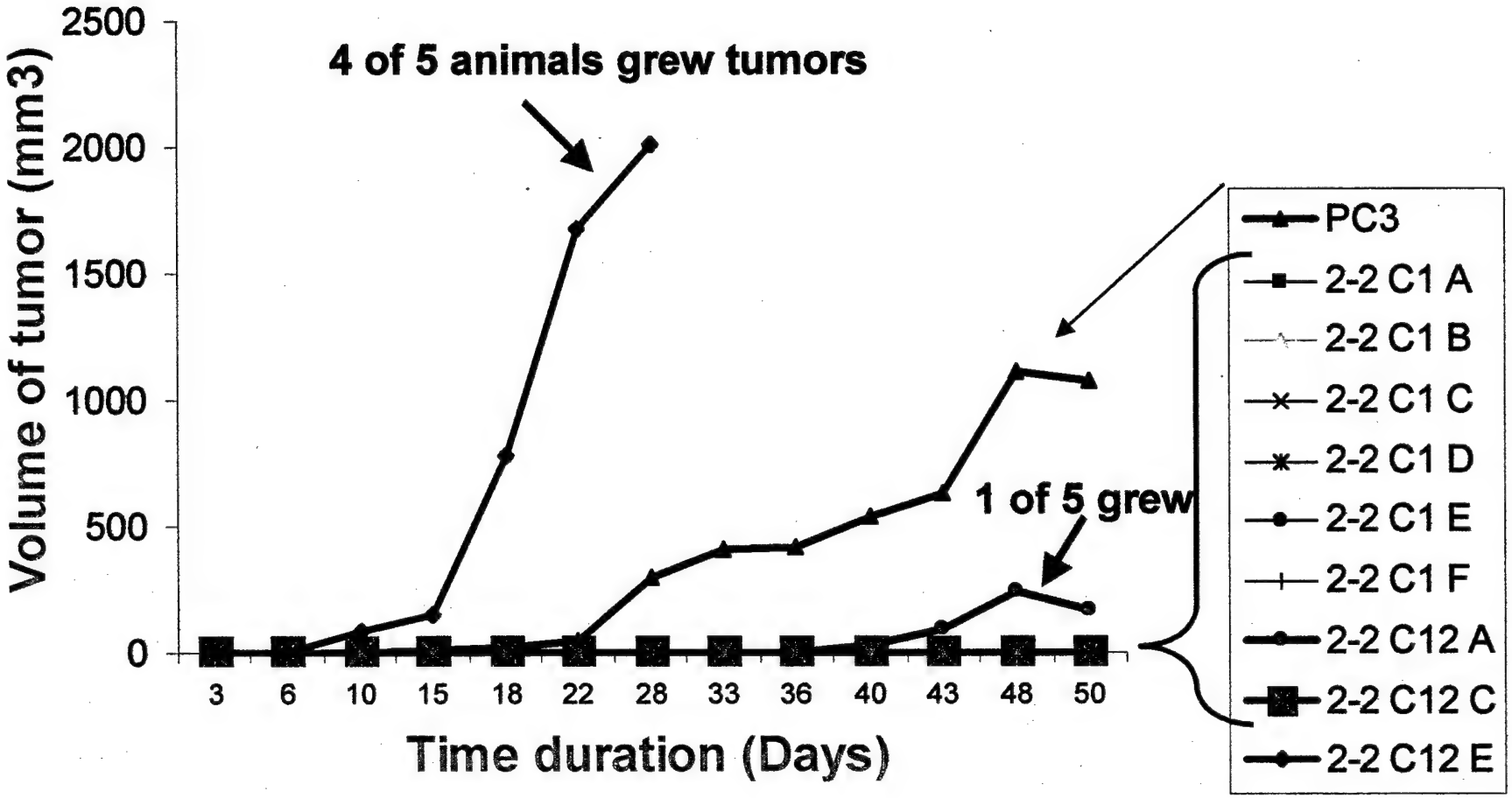


Figure 2. Growth of PC-3 soft agar subclones in nude mice



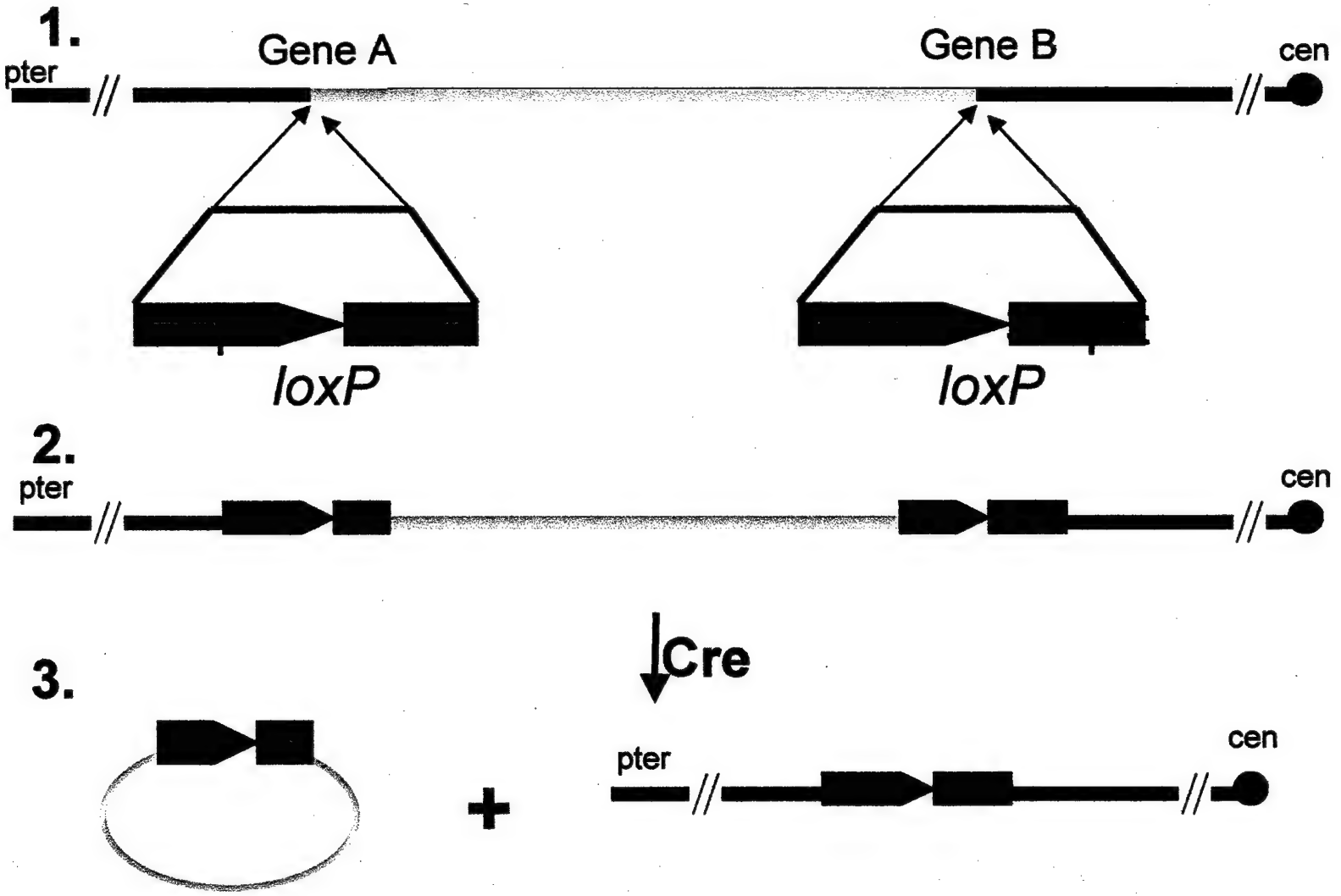


Figure 3. Site directed deletion of the Y chromosome.

1. **Two plasmids containing parts of the HPRT gene are inserted at specific gene A and B on Yp.**
2. **The resulting chromosome has the configuration with both puro and neo resistance.**
3. **Transient transfection with Cre recombinase results in the recombination of the two loxP sites. The intervening DNA is lost and the Y chromosome has a defined deletion.**

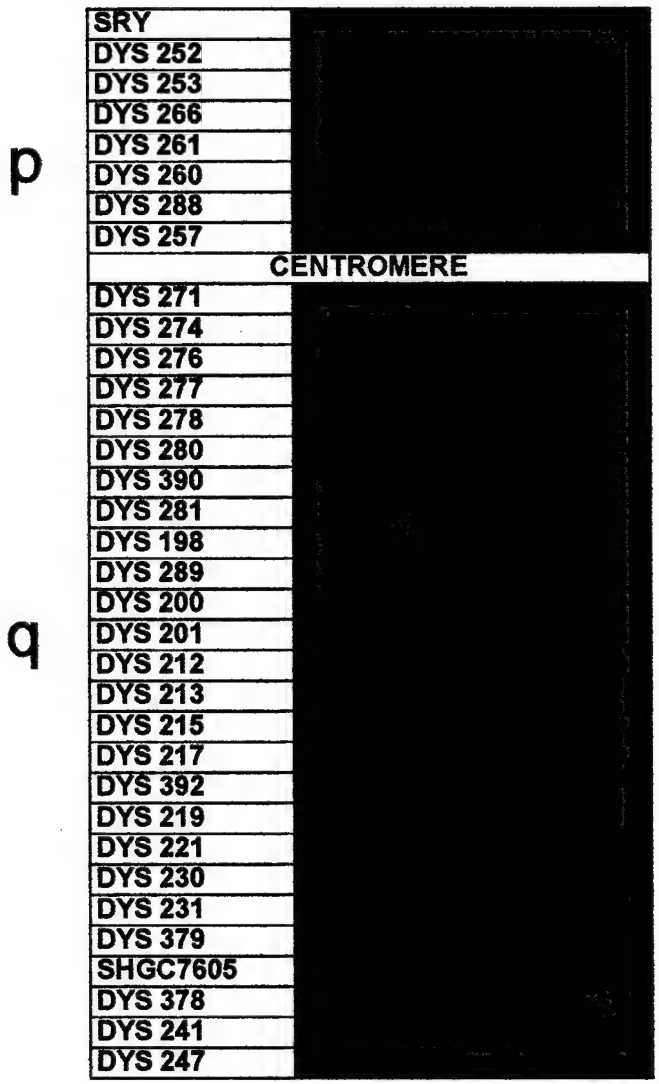


Figure 4. Summary of the smallest region for the Y chromosome tumor suppressor gene

Y chromosome suppresses the tumorigenicity of PC-3, a human prostate cancer cell line, in athymic nude mice.

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The loss of Y chromosome is the most frequent numerical chromosomal abnormality observed in human prostate cancer. In cancer, loss of specific genetic material can imply simultaneous loss of tumor suppressor gene (TSG). Mutations of classical TSGs including p53[1], Rb1[2] and p16[3] are rare in sporadic prostate cancer. Current evidence indicates that Y chromosome is lost in several other cancers including leukemia[4], bladder cancer[5], esophageal carcinoma[6], gastric cancer[7] and pancreatic cancer[8]. However, the significance of the loss of a sex chromosome in the development of different types of cancer is still unknown. To address this question in human prostate cancer, we used a functional approach by complementing Y chromosome in PC-3, a human prostate cancer cell line lacking a Y chromosome. Our results show that the addition of Y chromosome suppresses tumor formation by PC-3 in athymic nude mice. This observation suggests the presence of a TSG on the Y chromosome.

Prostate cancer is the second leading cause of cancer deaths in American men[9]. Cytogenetic studies have shown several chromosomal imbalances occurring in prostate cancer. These studies have indicated loss of chromosomal material from 1q[10], 5q, 6q, 7q, 8p, 10q, 13q, 16q, 17q, 18q, Xq (reviewed by Brothman et al. 1999) and Y[12] in prostate cancer. One study reported loss of short arm of Y chromosome in 35% of prostate tumor samples[13]. Because the loss of Y chromosome is common in prostate cancer cells and not in the normal stromal

cells[14], we hypothesize that loss of Y chromosome plays a significant role in the genesis/progression of the cancer.

We directly tested for the suppressive effect of the Y chromosome by first tagging the human Y chromosome with a selectable marker. A Chinese hamster/human cell hybrid, GM (Coriell Institute for Medical Research, New Jersey) carrying a Y chromosome as the only human chromosome was used as the source as the Y chromosome. We successfully targeted histidinol (*hisD*) resistance gene to the *mic2 locus* on the Yp using the vector pHTtkM3 obtained from Christine Farr (personal communication). This vector has a 7.5 kb intronic fragment of MIC2 gene contained in pSV2his. The presence of *hisD* on the Y chromosome was detected by Tyramide Signal Amplification-Fluorescence in situ Hybridization (TSA-FISH) using 3.2 kb fragment containing *hisD* gene from the targeting vector pHTtkM3 (Fig.1). Later, the same metaphase was reprobed with a commercially available human chromosome paint specific for Y repetitive sequences. Several independent hamster/human cell hybrids with a tagged Y chromosome were established, but only three, pHTtkM3 1-5B, pHTtkM3 2-2C and pHTtkM3 2-6 B, were used in further experiments. The *hisD*-tagged Y chromosome was transferred to a recipient cell line using microcell mediated chromosome transfer (MMCT). PC-3 lacks complete Y chromosome[15] and is an appropriate cancer cell line for genetic complementation studies involving Y chromosome. Using the Chinese hamster/human cell hybrids we created, three independent

chromosome transfer experiments were done for PC-3. A total of twelve different PC-3 hybrids each carrying a Y chromosome was further analyzed.

To assess whether Y chromosome can revert the tumorigenic phenotype of PC-3, we tested the tumorigenicity of PC-3 hybrids in Balb/c *nu/nu* mice. The twelve PC-3 hybrids were injected subcutaneously at the dorsal flank at the rate of 2 million cells/animal. Out of 60 mice injected with twelve different PC-3 hybrids, tumor growth was apparent in only one mouse in contrast to control animals all of which developed tumors (Fig.2). None of the mice exhibited any signs of metastasis. In previous studies using a similar approach, chromosomes 12[16], 17q[17] and 10[18] were shown to suppress tumorigenicity of prostate cancer cell lines, DU-145, PPC-1 and PC-3 respectively. Portions of chromosome 5 also suppressed tumorigenicity of PC-3[19]. The insertion of chromosomes 2, 7, 8, 10, 11, 12 and 16 did not reduce the tumorigenicity of Dunning rat prostate cancer[20]. Similarly, chromosome 3 failed to suppress the tumor formation by DU-145[21]. Therefore, the tumor suppression we observed with Y chromosome is a nonrandom effect.

In contrast *in vitro* tumorigenicity did not reflect suppression of growth. The insertion of Y chromosome also did not influence the doubling time of PC-3 cells (data not shown). All twelve PC-3 hybrids plated at 1000 cells/ 60 mm plate, grew on soft agar suggesting that complementing Y chromosome in PC-3 did not have any impact on the anchorage-independent growth of the cell (Fig.3).

Subclones isolated from the soft agar were retested for *in vivo* tumor suppression. Only one of 9 subclones that were injected developed tumors. These data indicate that tumor suppression in nude mice is independent from *in vitro* growth suppression in this system.

Only one mouse of the five injected with a PC-3 hybrid, 2-6B E2, developed a tumor. This tumor retained all 34 markers we tested, but it is the only tumor that was observed. Similarly, all remaining clones except 2-2 C1 seemed to have an intact Y chromosome. The hybrids 2-2 C1, 2-2 C2 and 2-2 C3 had major deletions of the Yq (Fig. 4). Despite this loss on the long arm, the tumorigenicity of PC-3 was suppressed. We conclude that these regions are not critical for the tumor suppression potential of the Y chromosome. Thus, we have narrowed down the region that harbors TSG primarily to the short arm.

Our findings imply that Y chromosome plays a very important role in the development of prostate cancer since it can suppress tumor formation. The Y chromosome contains several hundred genes. It is also worth to note that in a deletion analysis on prostate cancer samples, loss of six genes were observed between the region Yp11.3 and Yq12.1[22]. From our study, all hybrids seemed to have retained the short arm of the Y chromosome. Furthermore, a study by Tricoli et al. showed that loss of Yp is more frequent than Yq in prostate tumor samples[13]. However, the same group noticed normal copy number for Y chromosome using touch preparation of tumor samples instead of paraffin-

embedded sections[23]. Few studies have reported gain of Y chromosome in prostate cancer[24]. If this gain was of functional significance, then with the insertion of Y chromosome into PC-3, we expect to see an even more aggressive growth behavior by the PC-3 hybrids. Since this was not the case with the PC-3 hybrids, our data do not support the presence of oncogenes on Y chromosome bur rather a tumor suppressor gene. A gene expression analysis provided clues that expression of certain Y chromosome specific genes are diminished in prostate tumors[25],[26]. In summary, these observations and our findings on the suppression of tumorigenicity by Y chromosome provide strong evidence in favor of a functional involvement of this sex chromosome in the development of prostate cancer.

Methods

Tagging Y chromosome

Chinese hamster/human hybrid cell line containing Y chromosome was maintained in MEM Eagle's medium supplemented with 15% Fetal Calf Serum. The cells were transfected with hisD containing targeting vector pHTtkM3. After transfection, the cells were grown in the selective medium (with no histidine) containing 5mM histidinol.

Detection of Y chromosome

Fluorescence *in situ* hybridization: FISH was done as described[27]. The probe for *hisD* was prepared as follows. A 3.2 Kb XhoI-EcoRI fragment containing *hisD*

was biotin-labeled by nick translation (Gibco-BRL). Tyramide Signal Amplification (TSA)-FISH was done on Chinese hamster/human hybrid with tagged Y chromosome following Schriml et al. procedure with minor modifications[28]. 4,6-diamidino-2-phenylindole was used as the counter stain. The slide was viewed using Ziess Axioscop fluorescence microscope and the image captured by Applied Imaging's Probe Vision. After washing and denaturing, the slide was reprobed with a Y chromosome paint (Vysis, Downers Grove, IL).

Microcell mediated chromosome transfer

MMCT was done following the protocol described with minor modifications[29]. Briefly, Chinese hamster/human hybrid with the tagged Y chromosome served as the donor and PC-3 as the recipient. The donor cells were treated with 0.06μg/ml of colcemid for 24-48 hours. Microcells were obtained after centrifugation at 15,000 rpm for 30 minutes at 34°C in the presence of 10 μg/ml of cytochalasinB. Microcell were resuspended in 100μg/ml of phytohemagglutinin-P (PHA) and later fused with PC-3 cells in the presence of 50% polyethylene glycol (PEG) and cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium. After 24-48 hours, the cells were put in selection using DMEM/F-12 (without histidine) containing 5 mM histidinol. Following 10-14 days in culture, the resultant hybrids were tested for the presence of Y chromosome.

Assays for tumor suppression

***In vivo* tumorigenicity assay**: All cells were injected subcutaneously in five weeks old male Balb/c *nu/nu* mice. Each cell line was injected into five animals. The tumor growth was measured twice weekly and tumor volume was calculated using the formula, (length*width^2)/2. Any tumor formed in the experimental group was aseptically removed and expanded in DMEM/F-12 (nonselective) for further analysis. Those mice that did not form tumors were monitored for three months.

***In vitro* assay**: Both PC-3 and the hybrids were seeded on 60mm soft agar plates (n=4) at a density of 1000 cells/plate. The soft agar plates have a base layer containing 0.4% agarose, 10% DMEM and 10% fetal bovine serum and a top layer consisting of 0.24% agarose. The cells were fed after 1-1.5 weeks and scored after two weeks using p-iodonitrotetrazolium violet as the dye and counted using the software GelExpert (Nucleotech Corporation, San Mateo, CA).

Characterization of the hybrids

All hybrids injected were assayed for the presence of 34 Y chromosome specific markers. Each 20μl polymerase chain reaction (PCR) consisted of 120 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl, pH8.3, 1.5–3.0 mM $MgCl_2$, 200 μM dNTPs, 10 ng of each primers and 0.75 U of Taq polymerase (Gene Choice, Frederick, MD). Stepdown program was used for amplification. PCR products were visualized on 1.5% agarose gel by ethidium bromide staining.

Acknowledgements

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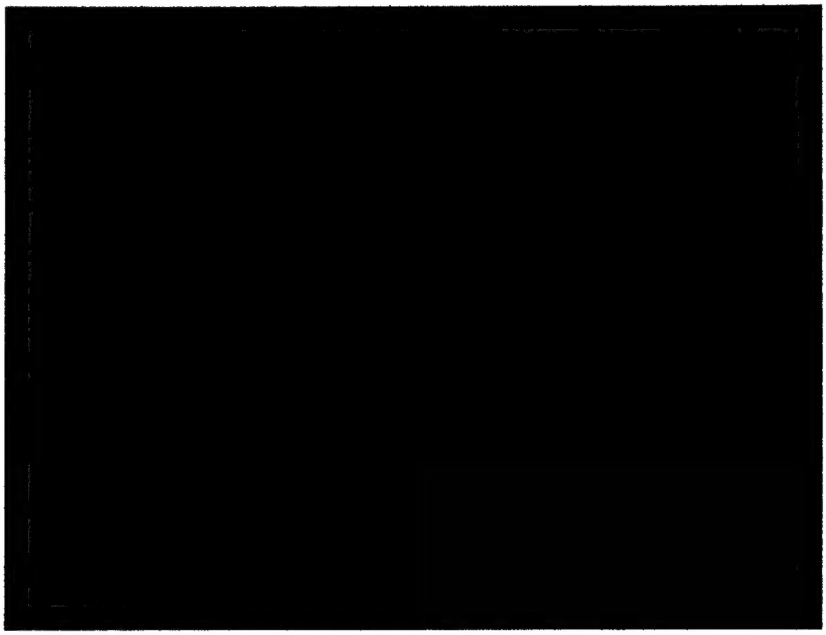
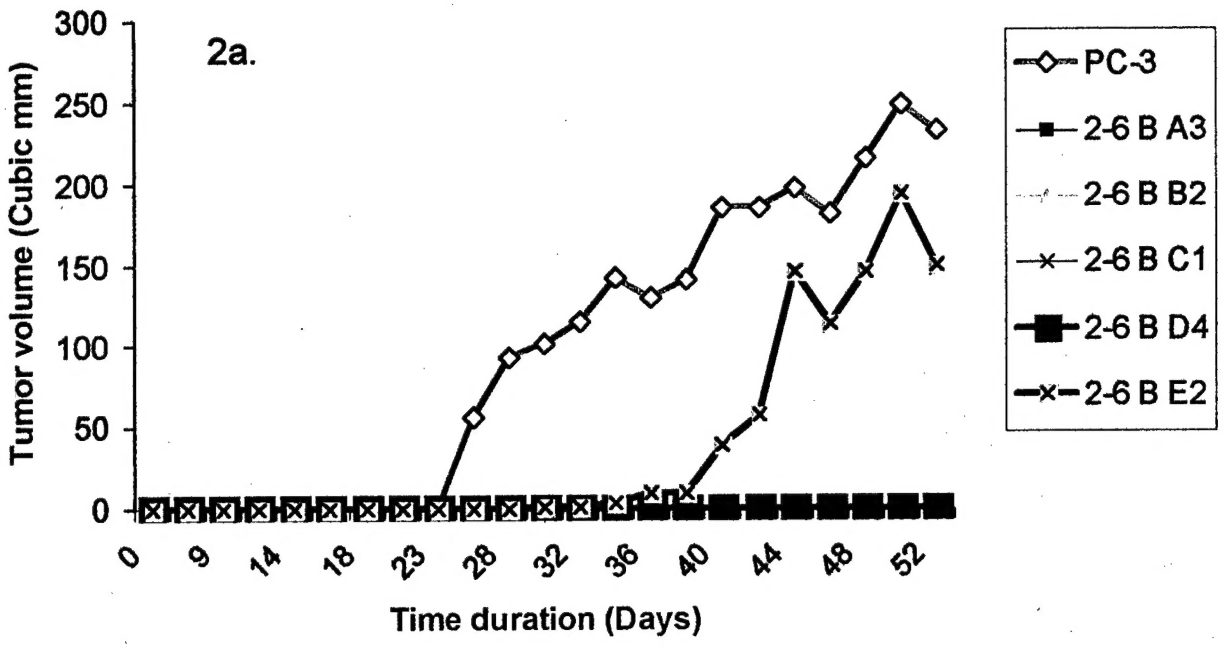


Fig.1. Chinese hamster/human hybrid cell showing the marker hisD (green) targeted to Y chromosome. Inset shows FISH done subsequently using a Y chromosome paint (pink) on the same metaphase.



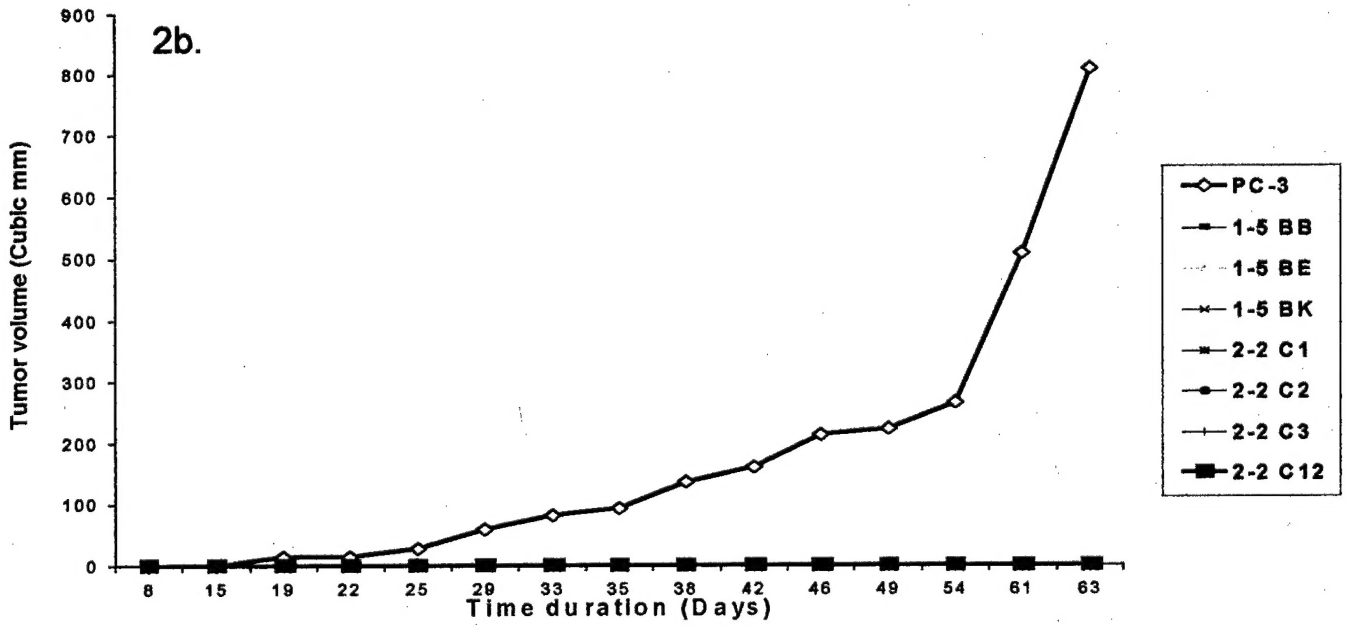


Fig.2a. Human Y chromosome suppresses the tumor growth of PC-3 in nude mice. Out of 25 mice injected with PC-3 hybrids, only one mouse injected with 2-6 B E2 (n=5) formed tumor. 2b. Suppression of tumor growth by seven independent PC-3 hybrid clones (n=5).

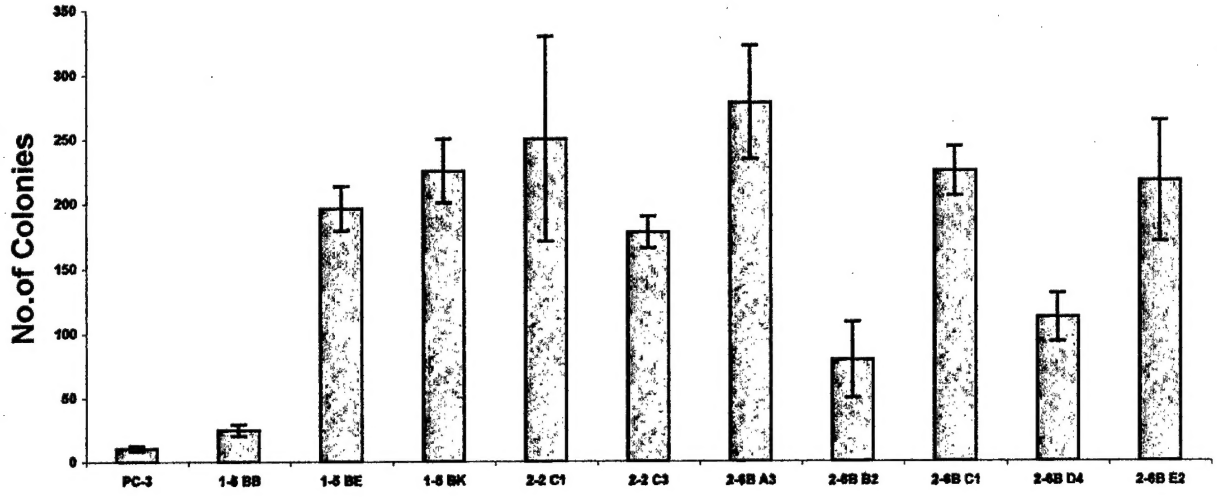


Fig. 3. Presence of Y chromosome did not influence the anchorage-independent growth of PC-3. All PC-3 hybrids, plated 1000 cells/ dish grew well on soft agar.

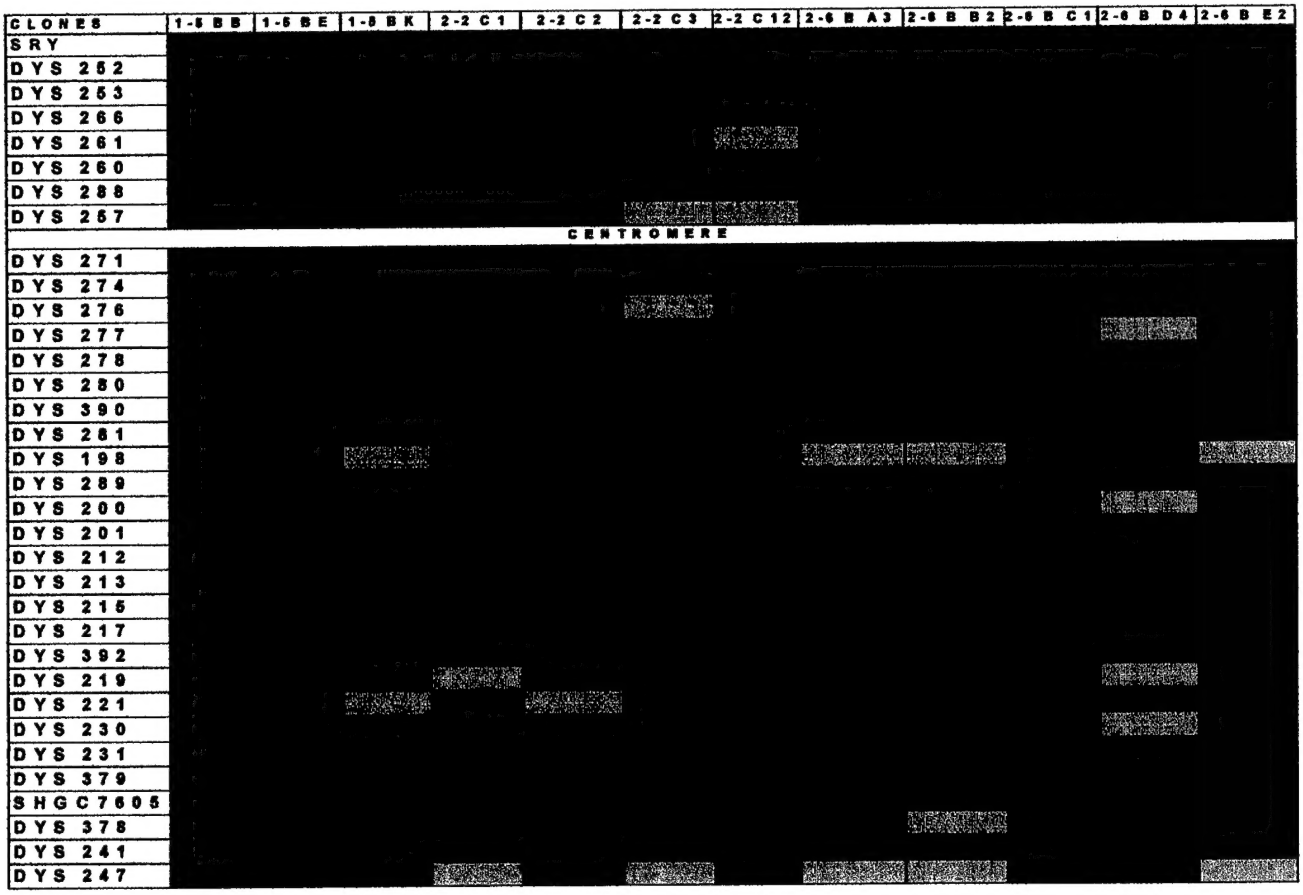


Fig. 4. PC- 3 hybrids exhibited regional losses on the Y chromosome. The clones retained an intact short arm (Yp) most of the times. The hybrid 2-6 B E2, which grew tumor in nude mouse retained all 34 markers tested.